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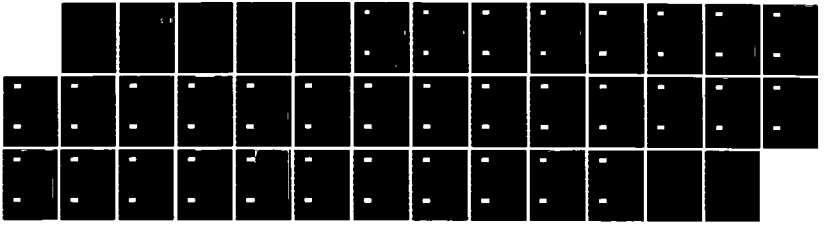

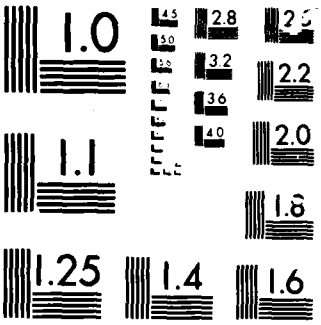

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MECHANISMS OF DRUG TOXICITY AND ANTAGONISM

Final Report

James L. Way, E. Leong Way, Toshiko Imamura, Mark Jones,
Ing K. Ho, Evelyn L. McGown, Nancy Lee.

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Drug antagonism, cyanide, organophosphorus compounds, heavy metals, sulfurtransferases, oxygen.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The initial topic of this symposium emphasizes that the toxicity ascribed to a chemical may be due to a minor contaminant in spite of the fact that the target organ for the toxicity may be quite discrete. The rest of the symposium deals with the antagonism of chemical intoxication. This area of research is still at a relatively primitive stage, as there are very few chemicals where specific antidotes have been designed to antagonize their toxic effects. For example, it would be difficult to enumerate more than a handful of chemical toxicants which can be antagonized by specific antidotes to protect against 6 LD50 doses. Some of these studies which are presently

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#20. ABSTRACT (cont'd).

being presented represent some of the highlights of chemical antagonism. In each of these discussions on chemical antagonism, the approach to develop a specific antagonist has been based on a reasonable scientific basis. More important, the groundwork laid out on these chemical antagonisms have formed the impetus to seek further improvements by gaining greater insight into the mechanism of chemical intoxication and its antagonism. Some of these improvements are based on improving the biologic disposition of the antidote so that the antagonism can be conducted more safely and conveniently. Also, although some of the chemical antagonisms were based on a scientifically sound basis at that time, its mechanism of antagonism presently must be viewed from a different perspective even though the efficacy of the chemical antagonists remain undiminished. And lastly, in chemical poisoning and its antagonism, one should not lose sight of the fact that the ultimate effect of an antidote is not demonstration in in vivo studies, but a verification of its efficacy in the intact animal. Very often, the in vitro data does not correlate with the in vivo effects and in those instances every effort should be made to investigate the in vitro data with the intact animal.

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MECHANISM OF CHEMICAL POISONING AND ITS ANTAGONISM

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The selection of this symposium topic for the Western Pharmacology Society meeting is quite fitting, as the first American toxicologist, Dr. James Blake, ini tiated many of his pioneering studies on the mechanism of chemical intoxication and its antagonism on the west coast many years before John Jacob Abel. If Dr. Abel is considered as the father of pharmacology, then Dr. James Blake should be con sidered the father of American toxicology. His toxicodynamic approach was con ducted in a rational scientific manner, and when one considers that these studies were conducted approximately 150 years ago, it must be viewed with great awe.

The first speaker today emphasizes that the toxicity ascribed to a chemical may be due to a minor contaminant in spite of the fact that the target organ for the toxicicity may be quite discrete. The rest of the symposium deals with the antagonism of chemical intoxication. This area of research is still at a relatively primitive stage, as there are very few chemicals where specific antidotes have been designed to antagonize their toxic effects. For example, it would be difficult to enumerate more than a handful of chemical toxicants which can be antagonized by specific antidotes to protect against 6 LD_{50} doses. Some of these studies which are presently being presented represent some of the highlights of chemical antag onism. In each of these discussions on chemical antagonism, the approach to develop a specific antagonist has been based on a reasonable scientific basis. More important, the groundwork laid out on these chemical antagonisms have formed the impetus to seek further improvements by gaining greater insight into the mecha nism of chemical intoxication and its antagonism. Some of these improvements are based on in depth studies on the chemistry of the toxicant whereas other studies are based on improving the biologic disposition of the antidote so that the antagon ism can be conducted more safely and conveniently. Also, although some of the chemical antagonism were based on a scientifically sound basis at that time, its mechanism of antagonism presently must be viewed from a different perspective even though the efficacy of the chemical antagonist remain undiminished. And lastly, in chemical poisoning and its antagonism, one should not lose sight that the ultimate effect of an antidote is not demonstration in in vitro studies, but a verification of its efficacy in the intact animal. Very often, the in vitro data do not correlate with the in vivo effects and in those instances every effort should be made to investigate why the in vitro data do not relate with the intact animal.

This symposium today initiates what is hoped to be a series of symposia on the mechanism of chemical intoxication and its antagonism. It is a fruitful area of research and, as we will see this evening, it is a productive area in which to con centrate one's investigative efforts.

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PHARMACOLOGIC AND TOXICOLOGIC BASIS OF CYANIDE ANTAGONISM

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It seems most appropriate to discuss the pharmacology and toxicology of cyanide at the Western Pharmacology Society meeting, as investigation of the biologic basis of cyanide and its antagonism was initiated in this country by Dr. James Blake (1,2) truly a westerner and one who certainly could be considered the father of American toxicology. Dr. Blake was a student of Francois Magendie and continued the interest of his mentor in attempting to elucidate the mechanism of cyanide intoxication and ints antagonism. He was the first to report that the onset of action of cyanide varied with its route of administration and, more important, Dr. Blake indicated that artificial respiration was effective in antagonizing the lethal effects of cyanide. His research on cyanide was initiated at the University College in London, but subsequently he migrated to the United States. He had an illustrious career in the west in the conceptual development of a rational basis for the mechanism of intoxication of various chemicals.

Much of our interest in the lethality of cyanide has been concentrated on its rapid onset of action; however, the most common occurrence and most widely distributed toxicologic problem with cyanide are associated with its chronic low level toxicity from various dietary, industrial and environmental factors.

MECHANISM OF INTOXICATION: The lethal effects of cyanide have been frequently attributed to a histotoxic anoxia produced by the inhibition of cytochrome oxidase, the terminal oxidase of the mitochondrial electron transport respiratory chain (3,4). In massive cyanide poisoning where the dose of cyanide greatly exceeds the amount necessary to inhibit cytochrome oxidase, it seems reasonable that the mechanism of toxicity is probably much more complex than a single biochemical lesion, as various other enzyme systems and biological intermediates, particularly metalloenzymes and critical Schiff base intermediates, are involved. The mechanism of cyanide intoxication will not be discussed here due to space limitations; however, it should be pointed out that there are many enzymes which are either sensitive or equally as sensitive to cyanide as cytochrome oxidase (5).

PHARMACOLOGIC BASIS FOR THE TREATMENT OF CYANIDE INTOXICATION: The pharmacologic basis for the treatment of cyanide intoxication was developed 50 to 150 years ago. Although the mechanism presently perceived may be viewed from a different perspective, the antidotes remain essentially the same with minor modifications. The antagonism of the lethal effects of cyanide by ventilation was first reported by Blake (1,2). In 1933, K.K. Chen reported the use of an antidotal combination of amyl nitrite, sodium nitrite and sodium thiosulfate (6). The mechanism proposed for cyanide antagonism was to sequester cyanide either by nitrite oxidation of methemoglobin so that the latter compound would interact with cyanide to form cyanmethemoglobin (6), or to sequester cyanide to form a stable metal

complex with cobalt (7). In addition to the sequestration of cyanide, it could be detoxified by its metabolism to thiocyanate (8-11). The various antagonists to cyanide are classified arbitrarily into three classes, even though the basis for these three classifications may be viewed from a different perspective at the present time.

A. Sequestration of Cyanide

1. Nitrites: The nitrites were employed in order to generate methemoglobin, as cyanide could interact with methemoglobin to form cyanmethemoglobin, and also methemoglobin could reactivate cyanide-inhibited cytochrome oxidase. The rationale for the use of amyl nitrite was that this volatile compound could be immediately inhaled while sodium nitrite was being prepared for intravenous administration. Because of the relative slow rate of formation of methemoglobin by sodium nitrite, this led to the development of more rapid methemoglobin formers, 4-dimethylaminophenol, DMAP (12).

Recent investigation suggests that the methemoglobin forming properties of sodium nitrite may play very little, if any, role in the therapeutic antagonism of the lethal effects of cyanide. When methemoglobin formation by sodium nitrite was prevented by methylene blue pretreatment, sodium nitrite either alone or in combination with sodium thiosulfate still protected equally as effectively against the lethal effects of cyanide (13). This does not infer that methemoglobin formation per se cannot play a role in antagonizing the lethal effects of cyanide, but to indicate that sodium nitrite formation of methemoglobin does not play a role on a therapeutic basis in antagonizing the lethal effects of cyanide. This has important conceptual and practical implications, for if sodium nitrite is exerting its therapeutic effect by a mechanism other than methemoglobin formation, this provides an opportunity for studying alternative mechanisms and for the development of a potentially new class of cyanide antagonist. Logically, an alternative explanation for cyanide antagonism by the nitrites is to investigate other pharmacologic properties of the nitrites. Since the nitrites are known to be potent vasodilators, then the vasogenic properties of these drugs were investigated as the potential mechanism of cyanide antagonism.

This prompted the investigation of various vasogenic compounds including the α-blocking adrenergic agents as cyanide antagonists. The α-adrenergic blocker, phenoxybenzamine, in combination with sodium thiosulfate, was found to be as effective as the sodium nitrite-sodium thiosulfate combination in protecting against the lethal effects of cyanide (15,16). It should be emphasized that phenoxybenzamine has no antidotal properties alone; however, it produces a striking potentiation when in combination with sodium thiosulfate which is equivalent to the nitrite-thiosulfate combination. Also, it should be noted that the antidotal effect of phenoxybenzamine can be reversed with the α-agonist, methoxamine. Of all the other autonomic agents and vasodilators examined, only the ganglionic blocking agents and α-adrenergic blockers were noted to exert an antidotal effect (16). The rationale why sodium nitrite and only certain other vasodilators should protect against the lethal effects of cyanide is being intensively investigated by various laboratories at the present time. It should be pointed out that chlorpromazine similarly can protect against the lethal effects of cyanide and the mechanism of protection is similar to phenoxybenzamine (17). It should be emphasized that the mechanism of chlorpromazine antagonism of cyanide intoxication was found to be related to the α-adrenergic blocking property of this phenothiazine rather than to hypothermia.

2. Cobalt Compounds: Cobalt compounds are known to form stable metal chelates with cyanide, and were used on this basis over ninety years ago to antagonize cyanide poisoning (7). There has been renewed interest in cobalt and more recently in hydroxocobalamin (18) and cobalt-EDTA (19). Cobalt-EDTA has recently been the preferred cobalt compound, as it was hoped that many of the toxic effects of cobalt ion could be minimized by administering this compound as an EDTA chelate.

B. Biotransformation of Cyanide

Rhodanese was described over 50 years ago (20). This enzyme, widely distributed in the body (8), catalyzes the detoxification of cyanide to thiocyanate and has been intensively investigated (10,11,21). There are two sulfurtransferases which may play a role in the detoxification of cyanide and they are thiosulfate and mercaptopyruvate sulfur transferase. Although these two sulfur transferases can detoxify cyanide to thiocyanate, their enzymic mechanism of detoxification, organ distribution and subcellular distribution are different.

In addition to the sulfurtransferases, Westley and his associates (11) have proposed a provocative hypothesis which adds intriguing perspective to the mechanism of cyanide detoxification. They proposed that this detoxification reaction can be derived from a sulfane sulfur pool which can react with cyanide. There are various biologic compounds which contain a sulfane sulfur and it is believed that these compounds rapidly equilibrate in the intact animal as a sulfane pool which can react with cyanide. A source of sulfane sulfur can be derived from cysteine by mercaptopyruvate which can then react with cyanide. Rhodanese not only can catalyze the trans of sulfur to cyanide but also can interconvert all the various sulfur sulfanes. A sulfane carrier mechanism was proposed as albumin and this serum albumin sulfane sulfur carrier complex then can react with cyanide. In vivo pharmacokinetic data are consistent with the development of a serum albumin sulfane carrier complex, as the conversion of cyanide to thiocyanate in dogs was found to occur predominantly in the central compartment with a volume distribution which approximates that of blood volume (22). Furthermore, the reaction rate of the sulfur albumin complex in vitro (11) would be consistent with its role in the detoxification of cyanide in vivo (22).

C. Oxygen

Since cyanide is believed to inhibit cytochrome oxidase (3,4) there does not appear to be a rational basis for employing oxygen to treat cyanide intoxication. Oxygen alone has only minimal effects in antagonizing cyanide intoxication; however, when it is employed in combination with sodium nitrite-sodium thiosulfate, a striking potentiation is noted (23,24). It should be pointed out that oxygen does not potentiate the effects of sodium nitrite and it has only relatively minimal effect in combination with sodium thiosulfate (23,24). Its protective effect is observed not only prophylactically but also therapeutically (24). This indicates that oxygen is not an adjunct therapy, but an integral part of cyanide antagonism. These beneficial effects of oxygen over air in cyanide intoxication have also been demonstrated physiologically by reversal of EEG changes (25). Also, the beneficial effect of oxygen over air in antagonizing cyanide intoxication can be demonstrated biochemically, e.g., decrease in cytochrome oxidase inhibition (26) and increase in glucose oxidation (27).

D. Treatment

Since there are various efficacious cyanide antidotes, there are differences of

opinion on which is the most efficacious antidotal combination in treating against cyanide intoxication. The treatment of cyanide poisoning can be separated into various categories.

1. Supportive Treatment: As indicated earlier, the supportive treatment cannot be overemphasized. Dr. James Blake (1,2) reported over 140 years ago that ventilation of the animal would enhance the protection against cyanide intoxication. Although there are many very effective antidotes available, the general supportive treatment should not be ignored and may be life-saving. The value of general supportive treatment (28) is particularly emphasized in a well documented study on cyanide poisoning (27) in which a man reported to have ingested 600 mg of potassium cyanide was successfully treated by supportive treatment alone.

2. Oxygen-sulfur Sulfane-methemoglobin: The classic antidotal combination of nitrite-thiosulfate is still one of the most effective treatment of cyanide poisoning (6), particularly when administered in combination with oxygen (23,24). Care must be taken in the administration of this combination, as it can be lethal in infants (29). There are advocates for the use of the rapid methemoglobin former, DMAP, in place of sodium nitrite, because of the rate of methemoglobin formation (12). Since we now know that the mechanism of therapeutic antagonism of cyanide intoxication by sodium nitrite is probably not related to methemoglobin formation, the rationale for the use of DMAP-thiosulfate in cyanide poisoning over nitrite-thiosulfate remains to be established.

3. Cobalt: Although cobalt-EDTA (19) is employed rather widely in the United Kingdom, Europe and the Scandinavian countries for the treatment of cyanide poisoning, caution should be emphasized with regard to its use. There have been clinical reports of severe cardiac arrhythmias of both auricular and ventrical origin. There is concern with the use of cobalt EDTA in clinical studies as serious signs of cardiac toxicity are being reported (30).

D. General Assessment:

The nitrite-thiosulfate treatment of cyanide poisoning, particularly in combination with oxygen, is still one of the most efficacious antidotal combinations for the treatment of cyanide poisoning. Objections to its slow rate of methemoglobin formation and presumed slow onset of action is conceptually erroneus, as sodium nitrite in combination with sodium thiosulfate can protect against cyanide intoxication without methemoglobin formation. There have been proponents for replacing sodium nitrite with a rapid methemoglobin former, DMAP, but whether DMAP is more effective than sodium nitrite in combination with sodium thiosulfate has still not been clearly established. With regard to the use of cobalt-EDTA, there should be some reservation with its use, primarily because of clinical reports on the severe cardiac toxicity from its use. It is anticipated that in the very near future there will be safer and possibly more effective cyanide antidotal combinations, particularly since there now is a greater amount of basic scientific information to aid in the rational design of more effective cyanide antagonists.

ACKNOWLEDGEMENTS: This work was supported by funds derived from NSF, NIGMS, NIEHS, and USAMRDC.

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PROTECTIVE EFFECT OF DRUG METABOLISM INDUCER AND INHIBITOR ON O,O,S-TRIMETHYL PHOSPHOROTHIOATE INDUCED DELAYED TOXICITY IN RATS

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O,O,S-Trimethyl phosphorothioate (OOS) is an impurity present in a number of widely used organophosphorus insecticides. For example, it is present in varying amounts in technical grades of malathion (1-3), phenthoate (1), acephate (2) and fenitrothion (4). All of these organophosphorus insecticides are generally regarded as safe, having rat oral LD_{50} values of 500 mg/kg or greater (5).

OOS has been shown to potentiate the acute toxicity of malathion and phenthoate (1). In addition, at low doses, OOS causes an unusual delayed toxicity in rats without producing typical cholinergic signs (6-8). The signs of delayed toxicity are weight loss and red staining around the nose, mouth and eyes. Electron microscopic observations have indicated that the morphology of Clara cells, the non-ciliated bronchiolar epithelial cells, were altered after OOS treatment to rats (9). Hence, attempts were made to examine whether the lesion could be quantitated using biochemical parameters. It is not known whether the delayed toxicity and/or lung lesion are due to the OOS molecule itself or to some metabolic product. This paper describes the protective effect of both metabolic inducer and inhibitor against OOS-induced toxicity, and demonstrates the direct correlation between lung damage and other signs of delayed toxicity.

METHODS. Male Sprague-Dawley rats (140-160 g) were purchased or bred in our own animal facility from animals obtained from Simonsen Laboratories.

Crude OOS was synthesized and purified according to the method of Umetsu et al. (2) and determined to be more than 99.9% pure by TLC and GLC. OOS was dissolved in corn oil (10 mg/ml) and a single dose, based on a preliminary toxicity study, of 20 mg/kg was given by intubation. Control animals received corn oil alone at 2.0 ml/kg.

All the rats were sacrificed on day 3 after OOS treatment unless otherwise specified. Piperonyl butoxide was dissolved in corn oil (250 mg/ml) and administered i.p. at 500 mg/kg either 2 h or 48 h prior to the OOS treatment. Phenobarbital was dissolved in saline (37.5 mg/ml) and given i.p. at 75 mg/kg for 4 consecutive days prior to OOS administration. SKF 525-A was dissolved in water (25 mg/ml) and given i.p. at 50 mg/kg 30 min prior to the OOS treatment.

The activity of lactate dehydrogenase (LDH) in the cell free supernatant of lung lavage fluid was assayed by the spectrophotometric method of Bergmeyer & Bernt (10). Protein was measured by the method of Sedmak & Grossberg (11). After collecting the lavage fluid, lung tissue enzymes were prepared by perfusing the lungs with 0.9% NaCl through the pulmonary artery until the lungs were

blanched. Each lung was homogenized. The homogenate was centrifuged at 5000 x g for 10 min and the supernatant was used to estimate the lung tissue LDH (10) and alkaline phosphatase (AP) (12).

RESULTS: The oral 28 day LD_{50} for OOS under the conditions of this study was 60 mg/kg with 95% confidence limits of 41-87 mg/kg. A single oral dose of 20 mg/kg (one third of LD_{50}) resulted in few acute cholinergic signs and all rats survived the dose at least 28 days; however, toxic signs were in evidence within 24 h and were present for at least 7 days. Typical signs of delayed toxicity were loss of body weight and red staining around the nose, mouth and eyes after OOS treatment. Since the signs of delayed toxicity of OOS were most apparent on day 3, pulmonary tissue enzymes (LDH and AP) and LDH level in bronchopulmonary lavage fluid was examined at this time.

As shown in Fig. 1(a), rats treated with OOS alone exhibited a significant weight loss as early as day 1 when compared with the corn oil treated groups which exhibited a gradual increase in body weight. The rats treated with phenobarbital prior to OOS exhibited an increase in body weight at the same rate as rats treated with corn oil alone, as depicted in Fig. 1(b). Pretreatment of rats with a small dose of OOS (5 mg/kg p.o.) for 4 days also prevented the weight loss induced by subsequently administered OOS (20 mg/kg) as shown in Fig. 1(b).

The rats treated with piperonyl butoxide 2 h prior to the OOS administration were also protected against the OOS induced body weight loss [Fig. 1(c)]. Qualitatively similar results were obtained by pretreatment with SKF 525-A. However, pretreatment with piperonyl butoxide 48 h befor OOS treatment failed to prevent the body weight loss [Fig. 1(c)].

When treatment prevented body weight loss the other signs of delayed toxicity, namely red staining around the nose, mouth and eyes, were absent or minimal.

Rats examined on day 3 following OOS treatment demonstrated a two fold increase in the activity of LDH in bronchopulmonary lavage fluid without producing a significant change in the protein concentration. The data on bronchopulmonary lavage fluid are presented in Table 1. The percentage of fluid recovered from the two successive lavages did not differ between treated groups. Fig. 2 depicts the time course of elevated lavage fluid LDH following oral treatment with OOS. Pulmonary injury, as assessed by this criterion, did not occur immediately after OOS treatment but rather was manifested between 3 and 5 days following the treatment. By day 7, lavage fluid LDH had returned to normal and was not significantly different from the control level. All the animals survived at least 7 days.

Rats pretreated with phenobarbital prior to OOS administration exhibited a level of pulmonary lavage fluid LDH activity which was not significantly different from that seen in rats treated with corn oil alone (Table 1). The protein concentration in the lavage was not altered by the treatment. Quantitatively and qualitatively similar results were obtained in the rats which were treated with small doses of OOS, SKF 525-A, and piperonyl butoxide 2 h prior to OOS treatment, as shown in Table 1, but not piperonyl butoxide at 48 h before OOS treatment.

The activity of LDH and AP in pulmonary tissue (Table 1) remained unaltered in the OOS treated rats when compared with the levels in the corn oil treated group. Treatment with other agents prior to the OOS administration had no significant effect on these activities compared with the OOS treatment alone (Table 1).

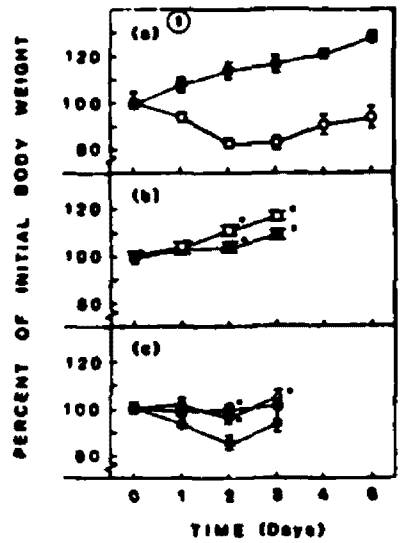

FIGURE 1: The effect of various pretreatment on the OOS (20 mg/kg p.o.) induced body weight loss. Each point represents the mean ± S.E. of 4-6 rats. ●, corn oil; O, OOS; □, OOS (5 mg/kg) for 4 days + OOS; ■, phenobarbital (75 mg/kg) for 4 days + OOS; ▲, piperonyl butoxide (500 mg/kg) 48 h + OOS; Δ, piperonyl butoxide (500 mg/kg) 2 h + OOS; ⬬, SKF 525-A (50 mg/kg) 30 min + OOS; (*) significantly greater than the OOS alone treatment group ($P < 0.05$).

Scanning electron microscopic examination of the surface of terminal bronchiolar airways revealed that oral administration of 20 mg/kg OOS caused extensive alteration of the morphology of terminal bronchiolar epithelium, i.e., decrease in number and increase in diameter of Clara cells. Pretreatment with phenobarbital, small doses of OOS, piperonyl butoxide or SKF 525-A all prevented OOS-induced morphological alterations of bronchiolar epithelium.

DISCUSSION: OOS is an impurity present in widely used organophosphorus insecticides and is capable of producing delayed death in rats (1-3). This toxicity could be due to either the OOS molecule itself or to some metabolic product. Many toxic agents appear to require metabolic activation before they produce cellular damage. Known examples include lung toxins (13), hepatotoxins (14) and carcinogens (15). The toxicity of chemicals which require metabolic activation can be prevented by the pretreatment of drug metabolism inhibitors and can be enhanced by pretreatment with inducers of mixed function oxidase activity.

The data presented clearly show that the treatment of rats with either a drug metabolism inducer phenobarbital or inhibitors piperonyl butoxide (2 h) or SKF 525-A prevented the signs of OOS induced delayed toxicity which includes weight loss, red staining around the nose, mouth and eyes, elevated level of bronchopulmonary lavage LDH and morphological alteration of terminal bronchiolar epithelium. This apparent paradox presumably must result from selective effects of each agent on different enzymatic processes. It is difficult to make accurate

TABLE 1: The effect of OOS and various pretreatments on pulmonary tissue LDH, AP and bronchopulmonary lavage fluid LDH

	A	B	C	D	E	F	G
Treatment parameter	Corn oil	OOS	Phenobarbital + OOS	Small dose of OOS + OOS	Piperonyl butoxide (2 h) + OOS	SKF 525-A + OOS	Piperonyl butoxide (48 h) + OOS
Pulmonary LDH (U/mg)	1.75±0.44[1]	1.51±0.14[1]	1.46±0.07[1]	1.61±0.06[1]	1.38±0.01[1]	1.45±0.12[1]	1.49±0.101
Pulmonary AP (U/mg)	5.29±0.24[1,2]	4.87±0.38[1,2]	5.49±0.33[1,2]	5.20±0.14[1,2]	4.40±0.33[2]	5.53±0.29[1]	4.95±0.33[1,2]
Percentage of lavage fluid recovered	90.3 ±2.3[1]	90.3 ±1.2[1]	93.8 ±1.3[1]	92.1 ±2.4[1]	95.6 ±1.1[1]	92.4 ±1.68[1]	93.3 ±2.41
LDH in lavage (U/100 ml)	6.6 ±1.4[1]	12.3 ±1.0[2]	7.4 ±1.2[1]	6.6 ±1.2[1]	5.2 ±0.7[1]	5.3 ±0.7[1]	11.3 ±1.7[2]
Protein in lavage (mg/100)	17.4 ±0.9[1]	16.0 ±1.3[1]	15.9 ±1.2[1]	14.4 ±0.9[1]	13.5 ±1.3[1]	15.9 ±2.1[1]	22.5 ±1.9[2]
N	5	6	4	4	3	6	4

Animals were treated as described in Methods. All the animals were sacrificed on day 3 after OOS treatment.

Results are expressed as mean ± S.E.

[1,2]Statistical comparisons between groups were done by one-way analysis of variance followed by Duncan's multiple range test. Treatment means in horizontal columns not followed by the same superscript are significantly different from each ($P < 0.05$).

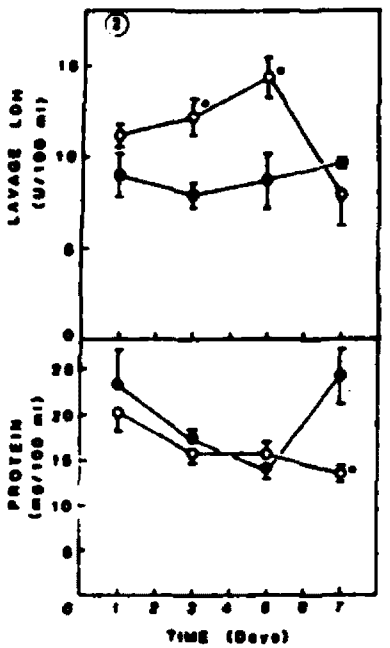

FIGURE 2: The effect of time after treatment with OOS on LDH activity and protein concentration in bronchopulmonary lavage fluid. Rats were treated with OOS (20 mg/kg p.o.) and lavage fluid were collected on days 1, 3, 5 and 7, as described in Methods. Control groups were treated with corn oil (2.0 ml/kg) and sacrificed at the same time with OOS-treated groups. N = 4-6; ●, corn oil; O, OOS: (*) significantly different from control of same day.

TABLE 2: The protective effect of various pretreatments against OOS-induced toxicity

Pretreatment	Phenobarbital	Small dose of OOS	Piperonyl butoxide (2 h)	SKF 525-A	Piperonyl butoxide (48 h)
Body weight	+	+	+	+	-
Red staining around the nose, mouth and eyes	+	+	+	+	-
Lavage fluid LDH	+	+	+	+	-
Morphological bronchiolar lesions	+	+	+	+	Not examined

+ indicates that there was a protection and - indicates that there was no protection.

predictions of the effect of a particular inducer or inhibitor on the toxicity of a compound that is metabolically activated in vivo. An inducer or an inhibitor may alter both toxifying as well as detoxifying metabolic pathways, and the net effect on toxicity of a compound will depend upon the final balance between the two types of pathways. In the present study, phenobarbital treatment gave a net induction of a detoxifying pathway, whereas piperonyl butoxide (2 h) or SKF 525-A treatment resulted in a net inhibition of an activation pathway of OOS.

There is precedence in the literature to support this conclusion (16). Studies on the lung toxin 4-ipomeanol reveal that its toxicity is reduced by both drug metabolism inducers and inhibitors. It was suggested that phenobarbital and 3-methylcholanthrene induce enzymes that detoxify 4-ipomeanol, whereas inhibitors such as piperonyl butoxide and pyrazole inhibit the activation processes of the compound. These findings are entirely compatible with the results of this present study, although we have not yet identified the pathways involved.

The results with piperonyl butoxide are of particular interest. It is well established that the compound is both an inhibitor and inducer of drug metabolizing enzymes. The inhibition by piperonyl butoxide is known to occur early after its administration, whereas its inducing effects of drug metabolizing enzymes in mice have been reported to occur 36-48 h (17). If this occurred in our study with rats, the piperonyl butoxide induced drug metabolizing enzymes could not be responsible for detoxification of OOS, and hence phenobarbital must be selectively inducing the enzyme system which is capable of detoxifying OOS. Another possibility is such that the enzyme level after piperonyl butoxide had returned to normal by 48 h and failed to afford any protection.

The effect of OOS was abolished by the prior administration of a small dose (5 mg/kg 4 days) of the compound. This could be either due to inhibition of activation pathways, as has been demonstrated with carbon tetrachloride (18), or induction of deactivation pathways. Pretreatment with OOS at 10 mg/kg for 4 days afforded no protection (data not shown).

Increased LDH and AP from lung tissue have been demonstrated following lung injury produced by nitrogen dioxide (19), ozone (20), beryllium (21) and benzo(a)pyrene (22) in rats. Furthermore, increased LDH activity in bronchopulmonary lavage fluid has been shown to be useful as a rapid screening test for detecting lung injury from chemicals which are pneumotoxic following inhalation or systemic administration (23,24). Our present investigation indicates that there was no change in pulmonary tissue LDH or AP on day 3. However, lung lavage fluid LDH activity was significantly increased on days 3 and 5 after OOS treatment. This result may suggest that the lung lavage fluid LDH activity is a more sensitive measure of pulmonary injury resulting from OOS treatment. Roth (24) investigated the effect of various pneumotoxicants which led him to postulate that the increase in the bronchopulmonary LDH activity might arise from diverse sources. However, the results of our study do not permit us to clarify the etiology of elevated LDH activity in bronchopulmonary lavage fluid.

The findings summarized in Table 2 indicate that pretreatment with phenobarbital, multiple small doses of OOS, piperonyl butoxide (2 h), or SKF 525-A protects against OOS induced body weight loss, and red staining around the nose, mouth and eyes. In contrast, piperonyl butoxide (48 h) had no effect on these parameters. Furthermore, direct correlations were found between these signs of toxicity and an increased level of lavage fluid LDH and morphological bronchiolar lesion. Thus, the lung could be a primary target organ in the manifestation of

delayed toxicity. The present study suggests that OOS induced delayed toxicity in rats was due to the formation of a toxic metabolite(s) which ultimately produces the lung lesion as determined by an elevated level of lung lavage fluid LDH and morphological alteration of terminal bronchiolar airways.

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ANTAGONISTS FOR TOXIC HEAVY METALS

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The antagonism of the toxic action of metals is generally based on the manipulation of the bonding preferences of the particular toxic metal, via transfer of metal to an appropriate metal chelate complex. These bonding preferences are determined by the position of the element in the periodic table, the oxidation state of the element in the specific compound present in vivo, and the donor atoms of the chelating agent. The manipulation of bonding preferences may be done directly, as is the case when a chelating agent is administered which can remove the metal from its in vivo bonding sites (Example: Pb^{2+} and EDTA, D-Penicillamine, BAL), or it may be done indirectly as when some other species is administered which can alter the metabolism of the toxic species in such a way as to render it less toxic [Example Vanadate + Ascorbic Acid (1)(2)] and interacting with the toxic metal to facilitate its removal. While chelating agents are the usual antagonists, every toxic metal tends to have certain aspects of its behavior that are unique, so that indirect methods may also be very effective with certain metals [e.g. zinc sulfate with the chronic copper intoxication of Wilson's Disease (3)].

DONOR PREFERENCES OF METAL IONS: The donor preferences of the metal ions are given by the hard and soft acid-base classification of Pearson (4). In this system we find that each oxidation state of a given metal can be classified on the basis of its coordination preferences towards "hard" Lewis bases such as F^- and $O^=$ or "soft" Lewis bases such as $S^=$ and $Se^=$. Most metal ions prefer one type strongly over the other although some are intermediate in character and others interact weakly with both types. A key feature of Pearson's theory is that these preferences also determine the rates with which one donor is replaced by another by a metal ion. A typical "hard" metal ion is Fe^{3+}, a typical "soft" metal ion is Hg^{2+}, while ions of intermediate character include Pb^{2+}, Cu^{2+}, and Zn^{2+}. Many of the toxic metal ions of interest are classified below (Fig. 1).

METAL PREFERENCES OF CHELATING AGENTS: The other side of donor preferences of metal ions is the acceptor preferences of the chelating agents. These are determined by the types of donor atoms present. For our purpose, we are concerned only with those chelating agents of sufficiently modest toxicity that they can be, or have been, administered to humans and animals and which act as antagonists to toxic metals under such circumstances. Common types of chelating agents which have been used in this fashion, and their coordination preferences are listed below (Fig. 2) (5).

It is important to note that none of the therapeutically useful chelating agents are specific for a given metal ion. Rather, each is capable of bonding to a fairly large number of ions with certain related patterns of behavior. The groupings above match the metal ions with the chelating agents with which they form stable metal chelate complexes. In the initial stages of the search for a metal ion

Approximate Character of Toxic Metal Ions

Soft	Intermediate	Hard
Hg^{2+}, Au^{+}, Pt^{2+}	Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+}	Fe^{3+}, Al^{3+}, Ga^{3+}
Pd^{2+}, Ag^{+},	Pb^{2+}, Sn^{2+}, Cd^{2+}, Cu^{+},	$VO_2^{+}(VO_3^{-})$, Be^{2+},
(platinum group ions)	As^{3+}, Sb^{3+}, Bi^{3+}, Tl^{+}, In^{+},	UO_2^{2+}, Sr^{2+}, Y^{3+}
	(many other transition metal ions)	Th^{4+}, Ce^{4+}, La^{3+}
		In^{3+}, Ra^{2+}, Pu^{4+}
		Lanthanide ions
		Actinide ions

FIGURE 1

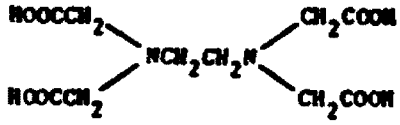
antagonist much time can be saved by a preliminary matching up of the toxic metal ion with the types of chelating structures with which it forms stable complexes (3).

Where measured values of the stability constants are available these can furnish a clue as to the best chelate antagonists for a toxic metal (26). In many cases, however, stability constants are not available and here the donor-acceptor patterns furnish a reliable guide for exploratory studies (5).

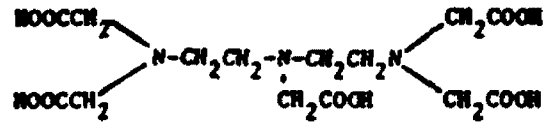
MODE OF ACTION: The chelating agent must get to the site where the toxic metal ion is held, often an intracellular one where it is bound to an enzyme or other molecule, successfully remove it from that site via formation of a complex which can then be disposed of, preferably by excretion. If the chelating agent is to be an effective antagonist for the toxic metal, the complex must be less toxic than the metal ion which it contains. For some chelating agents this does not occur because the bodily distribution pattern of the complexed form is different in a manner that allows the metal complex access to more sensitive sites than it could achieve as a simple ion. Some chelating agents cannot penetrate the cellular membrane, so they can tie up only extracellular metal. These work well with toxic metals which equilibrate rapidly between intracellular and extracellular bonding sites. Others can penetrate cellular membranes (10) and remove metal from intracellular sites. The mode of excretion of the complexed metal can have an important bearing on the net detoxification achieved. A major complication which can arise with water soluble metal complexes is the net transfer of metal into the kidneys with resultant damage to renal function. This occurs with lead, mercury, gold, cadmium and a large number of other species which damage the renal tubules.

TIME IS OF THE ESSENCE: For many toxic metals, delay in the initiation of chelate therapy results in an increasing level of permanent damage. Such permanent damage is especially well established in the case of certain organo-mercury compounds where, after a very short period, the clinical picture is largely independent of the rate of excretion of mercury. An analogous situation is commonly

CH_2CHCH_2OH SH SH BAL	$CH_2CHCH_2SO_3Na$ SH SH 2,3 dimercapto-propanesulfonate, sodium salt	HOOCCHCHCOOH SH SH 2,3 dimercapto-succinic acid	Prefer soft acids: Hg^{2+}, As^{3+}, Sb^{3+}, Bi^{3+} (6)

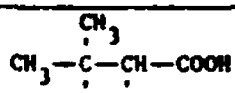


EDTA

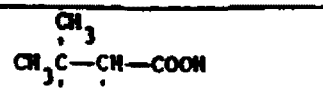


(and analogs) DTPA

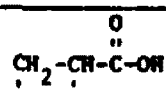
Prefer hard acids and intermediate acids: Al^{3+}, Ca^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Pu^{4+} (7)



D-penicillamine (8)(9)

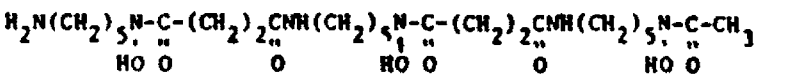


N-Acetyl,D,L penicillamine (10)



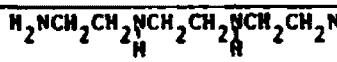
N-Acetyl-L-cysteine (11)

Coordinate best to soft and intermediate acids: Hg^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Au^{+}



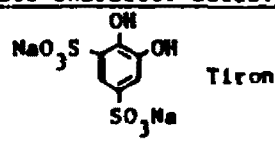
Desferoxamine

Prefers hard acids Fe^{3+}, Al^{3+}, Ga^{3+} (12)(13)



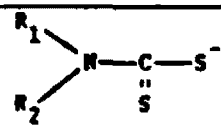
Triethylenetetramine and analogs

Prefers Intermediate character acids: Cu^{2+}, Ni^{2+}, (14)(15)



Tiron

Prefers hard and intermediate acids: Be^{2+}, UO_2^{2+}, Sb^{3+}, Bi^{3+} (16)(17)(18)(19)



Dithiocarbamates

Prefers soft and intermediate acids: Hg^{2+}, Cu^{2+}, Cd^{2+}, $Ni(CO)_4$ (20)(21)(22)

FIGURE 2

found in poisoning by many other heavy metals in which renal damage or damage to the central nervous system is a primary result.

NON-CHELATE ANTAGONISTS: Non-chelate antagonists cover a great variety of situations. Thus orally ingested thallium (Tl^{+}) can be absorbed by charcoal or prussian blue while it is still in the gastrointestinal tract (23) but these are useless once the thallium has been absorbed. Ascorbic acid is an excellent antidote for vanadate, presumably via its ability to reduce vanadate to the less toxic vanadium(IV)(VO^{2+}) (1,2). Zinc sulfate has been used to offset the toxicity of copper in Wilson's Disease and selenium (24) and zinc (25) are reported to reduce some of the toxicity of cadmium. Non-chelate antagonists for toxic metals are of special interest because they frequently allow a completely different approach to an otherwise difficult problem. There presently seems to be no reliable procedure for predicting the efficacy of non-chelate antagonists prior to experiment.

UNSOLVED PROBLEMS: Of the many unsolved problems in the area, the following are a small sampling.

Thallium: there is a need for a material which is an effective antagonist for thallium subsequent to its absorption from the gastrointestinal tract.

Actinides and Lanthanides: more effective agents are needed to enhance the rate of excretion of actinide and lanthanide elements, especially from the bone.

Aluminum: there is probably a need for an antagonist for the action of aluminum in Alzheimer's Disease.

Iron: an oral agent is needed which can induce the excretion of all of the iron administered to individuals who must have frequent blood transfusions over a period of years.

Cis-platinum: The expanding use of this compound in the treatment of cancer emphasizes the need for antagonists to offset its nephrotoxicity.

Gallium Nitrate: This compound, also used in cancer chemotherapy, will require an effective antagonist as its use expands.

Selenium: The widespread publicity given to the anti-cancer effects of trace amounts of this element will probably lead to an increase in the number of cases of selenium intoxication. There seems to be no agreement on effective antidotes for selenium intoxication.

Organometallic Compounds: The increasing industrial use of organometallic compounds must be accompanied by the development of effective antagonists for many of the most toxic of these substances.

In general, we also need to develop ideas that would allow us to purposely shift the excretion of a toxic metal from the sensitve renal route to the less sensitive fecal route.

SUMMARY: From a consideration of the donor preferences of toxic metals and the acceptor preferences of commonly used therapeutic chelating agents it is often possible to get a good estimate of the kinds of chelating agent which may be effective antagonists. The relative importance of extracellular vs. intracellular metal, however, introduces an important factor which can severely limit the utility of a

given chelating agent with a toxic metal with which it forms very stable complexes. In general an effective antagonist of a specific toxic metal will be a chelating agent which can gain access to the metal in vivo and which forms a very stable complex, of modest toxicity with that metal ion which is rapidly excreted without damage to the kidney or liver. Such effective antagonists are not known for all metals.

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BIOLOGICAL BEHAVIOR AND METABOLIC FATE OF THE BAL ANALOGUES DMSA AND DMPS

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BAL (2,3-dimercaptopropanol) was introduced in the early 1940s by Peters and his coworkers (1) after an intensive search for a substance that would counteract the severe burns caused by certain arsenical war gases. The name BAL is an abbreviation of "British antilewisite", a term applied to the substance after it proved effective against lewisite. BAL turned out to be effective not only in treating the vesicant action of arsenicals, but also in treating systemic toxic effects following their absorption. Subsequent investigations revealed that BAL was useful as an antidote to a number of heavy metal poisons. Although better antidotes have been found for most other metals, BAL is still the drug used in the USA for arsenic poisoning (2).

In this paper, I will review briefly the development of BAL and its properties and discuss two promising less toxic analogues. Because the antidotal efficacies and metal chelating properties of these compounds have been well covered in recent reviews (3,4), I will focus on their biologic behavior and metabolic fates.

DEVELOPMENT OF BAL: By the early 1940s, considerable evidence had accumulated supporting the hypothesis that trivalent arsenic and other heavy metals are toxic to biological systems because of their reversible sulfide formation with critical cellular sulfhydryl groups. In some systems, monothiols such as glutathione or cysteine afforded partial protection, but in others, e.g. the inhibition of pyruvate oxidase by lewisite, monothiols failed to protect, even when present in large excesses. This fact, along with other evidence, suggested that arsenic might combine with two sulfhydryls situated near each other within a single protein molecule to form a ring structure of greater stability than a dimercaptide derived from two separate monothiol compounds. Thus it was reasoned that in order to displace arsenic from its cyclic combination with proteins, the presence of competing dithiols which could form even more stable cyclic compounds would be necessary. The most likely dithiols were those with two or three carbons between the thiols which, combined with arsenic, would give rise to 5 or 6-membered rings, respectively. Numerous dithiol compounds were prepared and screened for their ability to protect pyruvate oxidase activity from inhibition by lewisite in vitro. Indeed, 1:2 and 1:3 dithiol compounds were effective (5,6). However, in a comparison of alkenedithiols containing 2 to 12 carbons between the thiols, those theoretically forming rather large rings with 9 to 13 atoms were about as potent as the 1:2 and 1:3 dithiols. Only those dithiols yielding 7 or 8-membered rings and those containing above 13 members had lower efficacy in vitro -- which the authors attributed to lower stability of the rings (6).

For the application of dithiol therapy to man, BAL was selected because it was among the least toxic of the compounds studied then and because its physical properties were suitable for the purpose intended at that time i.e. treatment of lewisite-contaminated skin. BAL is an oily smelly liquid. It is unstable in aqueous solution (unless ultrapure), and so the pharmaceutical preparation is in a peanut oil base and designed for intramuscular use. Unfortunately, BAL has other disadvantages including high toxicity and low therapeutic index. Half of patients receiving BAL experience such side effects as nausea, vomiting, and headache (3). Also because it is lipophilic, it penetrates rapidly into the intracellular space and, under some conditions, redistributes heavy metals into sensitive tissues such as the brain, rather than simply facilitating elimination (3,4).

METABOLIC FATE OF BAL: Little information is available on the metabolic fate of BAL. Most of the published work was reported in the late 1940s. When ^{35}S-BAL (approx. 0.5 mmol/kg) was injected into rats, 50 to 75% of the sulfur appeared in the neutral sulfur fraction of the urine within 24 h and most within 6 h (7,8). Thiols were present, but no unchanged BAL could be detected. Rabbits injected with BAL excreted a dithiol in the urine that corresponded to 20% of the injected BAL (9). The dithiol was spectroscopically similar, but not identical to BAL. Most intriguing was the fact that the urinary dithiol was effective against lewisite in vitro. In a subsequent study, 50 to 77% of labelled BAL injected into rats was excreted in the neutral sulfur fraction within 6 h (10). Approximately 4% of the sulfur appeared as inorganic sulfate. Paper chromatography revealed one major area of radioactivity and a minor spot presumably due to the inorganic sulfate. No unchanged BAL was detected. These reports collectively indicate that BAL is cleared rapidly from the body and is transformed into at least 2 metabolites including a dithiol with properties similar to BAL.

BAL is assumed to react with trivalent arsenicals in a 1:1 stoichiometric relationship to form a 5-membered ring. However, a 1:1 complex is not necessarily the only species for all metals. Gilman and coworkers (11,12) found that cadmium and mercury formed a 2:1 (BAL:metal) complex under physiological conditions.

BAL ANALOGUES: After BAL had been introduced, the search continued for a less toxic antidote that would be more appropriate for treating the systemic effects of arsenical poisoning. An early candidate was the glucoside of BAL (13). It was freely water soluble, much less toxic than BAL and possessed good therapeutic activity. Unfortunately, there were problems in obtaining pure and stable preparations, so the compound was abandoned. Research in this area was largely discontinued in the United States and Europe until recently. Two BAL analogues currently being studied are meso-dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS). DMSA was synthesized in England in 1949 (14) and DMPS was synthesized in both England (15) and Russia (16) around 1955. Since then, the metal chelating and antidotal properties of these compounds have been studied actively by investigators in the Soviet Union, Peoples Republic of China, and Japan. Both compounds are more water soluble and much less toxic than BAL, have similar chelating properties and are therapeutically effective when given orally (3). DMPS is known as unithiol in the Soviet Union where it is used to treat poisonings by such metals as lead, mercury, arsenic, and copper.

BIOLOGIC DISPOSITION OF DMPS AND DMSA. We have studied the disposition of ^{14}C-DMSA (UL) (gift from Johnson and Johnson) and DMPS-1,3-^{14}C (gift from Heyl & Co., Berlin) in male juvenile monkeys (Macaca mulatta). The animals were trained to accept restraint in a primate chair. Because they were familiar with the experimental procedures, stress was minimized and anestheisa was

never necessary. DMSA (0.16 mmol/kg) given intravenously was rapidly cleared from the blood stream with an initial half-life of approximately 45 min and most of the label rapidly appeared in the urine. The feces contained only traces of ^{14}C. When the compound was given orally, the peak plasma ^{14}C occurred at 1 1/2 to 2 h and declined rapidly with a concomitant appearance in the urine (Fig. 1). Fifty to 70% of the label appeared in the feces and 10 to 30% in the urine, most within 24 h. A small but consistent amount appeared in the breath as ^{14}C-CO_2. Table 1 summarizes the disposition of the ^{14}C in these experiments. Total recovery of label was over 80%. With DMPS, we obtained similar results both concerning pharmacokinetic behavior and final disposition (Table 2). In these experiments, labelled material in the blood appeared to be primarily, if not exclusively, in the plasma.

We have also examined the disposition of DMSA and DMPS at a higher dose in mice and hamsters. Oral DMSA was rapidly absorbed into the blood stream of both species and ^{14}C reached its peak concentration within 30 min (Fig. 2). Table 3 summarizes the disposition of this drug in the hamster. DMSA was rapidly absorbed and excreted in the urine, most of it within 6 to 12 h. Five percent appeared in the feces and the total excretion by 96 h averaged 87%. Thus gastrointestinal absorption of DMSA was much higher in the hamster than in the monkey. Our preliminary data suggest that DMPS is also well-absorbed by the hamster.

Our results are compatible with literature reports concerning other species. Rabbits rapidly absorbed ^{35}S-DMPS after subcutaneous injection and cleared the label with a half life of 60 min (17). Wiedemann and coworkers (18) in Germany found that 60% of an oral dose of DMPS was absorbed by dogs and plasma radioactivity reached a peak in 30-45 min. After an intravenous injection, radioactivity disappeared from the plasma with a half-life of 43 min and was quantitatively excreted via the kidney. This same group also reported on the basis of equilibrium dialysis experiments that most ^{14}C-DMPS was protein-bound. The fraction bound varied little over a concentration range of 50 to 500 µM. These results seem incompatible with the rapid renal clearance of the drug unless the binding constant is low and the dissociation rate extremely high. Gabard (19) reported a half-life of 19 min for ^{14}C-DMPS in the rat and the apparent distribution volume of ^{14}C was equivalent to the volume of extracellular water. Absorption was 30 to 40% when the drug was given orally. Because the percent absorption was similar over a 25-fold dose range (0.1 to 2.5 mmol/kg), he suggested that the drug diffused passively through the gut mucosa. He also speculated, on the basis of his pharmacokintic analyses, that DMPS is weakly bound to plasma proteins.

There are fewer reports on the disposition of DMSA. In the treatment of lead poisoning in rats, oral DMSA is 80% as effective as the same dose by intraperitoneal administration (20). This indirectly suggests that DMSA is well-absorbed by the rat. Whole body autoradiography after intravenous injection of a small dose of ^{14}C-DMSA (0.02 mmol/kg) into mice revealed at early times the highest levels of radioactivity in blood, lung, kidney, skin, and gastrointestinal contents (21). Most of the ^{14}C was eliminated within 24 h.

METABOLIC FATE OF DMSA AND DMPS: The metabolic fate of these compounds has not been well-characterized. Some work has been done on DMPS, but there are no reports dealing with DMSA at least in the Western world. Gabard & Walser (22) injected ^{14}C-DMPS intravenously into rats and found several radioactive peaks on thin layer chromatograms of the urine. They found no free DMPS (by R_f and color formation with nitroprusside) unless the sample had been acidified. The authors speculated that the acid had liberated DMPS from metal complexes.

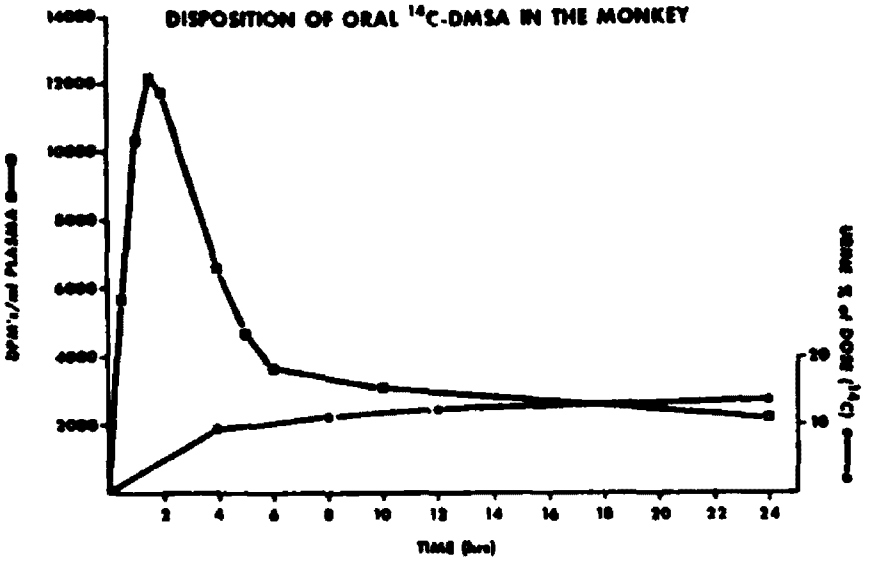

FIGURE 1: Radioactivity in blood and urine of the monkey following an oral dose (0.16 mmol/kg) of ^{14}C-DMSA.

TABLE 1: Biological fate of ^{14}C-DMSA (0.16 mmol/kg) in monkeys

	% Dose recovered	
	Oral (n. 4)	I.V. (n. 2)
Urine - 24 h	14.5	79.2
- 96 h	18.3	81.1
Feces - 96 h	64.9	0.3
$^{14}C-CO_2$	1.6	0.8
Recovery	84.8	82.2

(Note, however, that even after acid treatment, much of the radioactive material did not migrate with DMPS.) Urinary thiols corresponded to 18% of the dose. Despite the variety of radioactive peaks, they concluded that DMPS "is not involved in important metabolic reactions". Russian investigators (23) have reported on the basis of thin layer chromatography that ^{35}S-DMPS is oxidized slowly in rabbit serum in vitro to a tetrasulfide form via an intermediate that they presumed was a disulfide. When the compound was administered subcutaneously to rabbits, early urine samples (collected by catheter) contained both free DMPS and the tetrasulfide. Urine collected after 24 h contained only the tetrasulfide. They concluded

TABLE 2: Biological fate of ^{14}C-DMPS (0.13 mmol/kg) in monkeys

	% Dose recovered	
	Oral (n, 2)	I.V. (n, 2)
Urine - 24 h	21.8	77.1
- 48 h	25.0	78.1
Feces	69.3	0.8
^{14}C-CO_2	0.1	0.1
Recovery	94.3	79.5

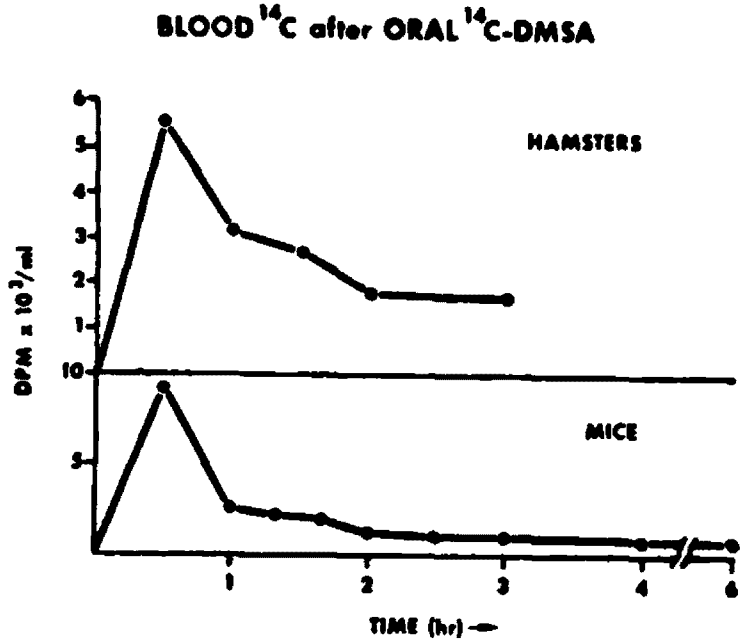

FIGURE 2: Radioactivity in blood of mice and hamsters following an oral dose (1.0 mmol/kg) of ^{14}C-DMSA. Each point represents the mean of 5 animals.

TABLE 3: Disposition of oral ^{14}C-DMSA in hamsters

Animal	% of dose Urinary ^{14}C 6 h	Urinary ^{14}C Total	Fecal ^{14}C	Recovery
1	45	76	7	83
2	60	89	9	98
3	27	77	4	81
4	36	83	1	84
Mean	42	81	5	86

DMPS is gradually oxidized in vivo as well as in vitro. The authors assumed the oxidized species to be a cyclic tetrasulfide. However, there are numerous theoretically possible structures for sulfides of this drug including linear or branched polymers.

We have begun investigating the metabolic fate of DMSA. We felt it likely that at least some of the compound was excreted unmetabolized, other than oxidation of its thiols to disulfides. Therefore we devised a method to analyze urinary DMSA. We determined total sulfhydryl groups in urine spectrophotometrically with the disulfide exchange reagent 4,4'-dithiodipyridine. This compound reacts quantitatively with thiols to form 4-thiopyridone, which absorbs strongly at 324 nm (24). We found that DMSA and DMPS reacted faster with this reagent than with Ellman's reagent, possibly because 4,4'-dithiodipyridine, in contrast to Ellman's reagent, is unionized under assay conditions. Urinary disulfides were reduced electrochemically at a mercury pool electrode by passing a 3.5 mA current through the cell until reduction of disulfide compounds was complete (as determined by serial removal of aliquots and sulfhydryl analysis). When necessary, reduced samples were partially purified by affinity chromatography with agarosebound p-mercuribenzoate. Sulhydryl compounds selectively bind to this material, thus allowing nonsulfhydryl compounds to be eluted. The sulfhydryl compounds, in turn, can be eluted by treating the column with a competing sulfhydryl compound such as cysteine. Cysteine was subsequently removed from the sample thiols by cation exchange chromatography. The resulting solution was acidified, extracted with ethyl acetate and the organic phase evaporated to dryness under nitrogen. N,O-bis-(trimethylsilyl)-acetamide (BSA) was added to form the (more volatile) trimethylsilyl (TMS) derivatives of compounds which possess acidic hydrogens and the resulting mixture was analyzed by gas chromatography. The TMS derivative of DMSA has been tentatively identified in gas chromatograms of hamster urine on the basis of peak retention time. (The retention time of the TMS derivative of DMSA was established by derivatizing and chromatographing pure samples of this compound.) For purposes of quantitation, mercaptosuccinic acid was added as an internal standard to the urine specimen after electrochemical reduction but before the purification procedure.

We found that the assay for DMSA and the spectrophotometric assay for total sulfhydryls (after reduction) gave identical results in urine samples from hamsters that had received oral or intraperitoneal DMSA (1.0 mmol/kg) (Table 4). In each case the amount of DMSA recovered was only slightly less (average = 87%) than the theoretical amount based on the radioactivity present and the specific

TABLE 4: Analysis of DMSA in hamster urine

	Oral				Intraperitoneal	
	1	2	3	4	1	2
^{14}C[1]	28.6	34.2	25.9	26.5	58.3	29.1
-SH[2]	26.8	31.0	23.2	21.3	53.4	25.6
DMSA	23.5	30.0	24.3	23.5	51.6	23.8
DMSA / ^{14}C%	82	88	94	89	89	82

Average DMSA/^{14}C = 87.3

[1]Theoretical concentration based on ^{14}C present.
[2]Assuming 2 sulfhydryls/mol.

activity of the dose. These data indicated that most, if not all, of the carbon chain of DMSA was excreted intact by the hamster. The sulfhydryls are oxidized, but we do not yet know how much of the oxidation occurs in vivo. Urinary thiols before reduction have been as high as 50% and as low as 10% of the post-reduction total. Much of the oxidation probably occurs in vitro before the sample is collected and analyzed, but DMSA that is not excreted in the early urine samples may be oxidized in vivo (23). These data give no clue about the oxidized form(s) of DMSA. Because DMSA quantitatively accounts for the assayable thiols, mixed disulfides (with endogenous thiols) are minor, if present at all. Although we consider it unlikely, we cannot eliminate the possibility that DMSA is excreted in a carboxyl conjugate. Acidification of the urine samples could be hydrolyzing carboxyl esters. However, little DMSA is taken up by tissues and such (enzymatic) conjugation reactions normally occur within the liver.

CONCLUSIONS: DMPS and DMSA remain largely extracellular, are cleared rapidly from the bloodstream, and are excreted via the kidneys. Absorption of oral doses is species-dependent, ranging (for DMSA) from 20 to 30% in the monkey and rabbit (Tillotson, unpublished observations) to >90% in the hamster. DMSA is excreted intact by the hamster, except for variable oxidation of its sulfhydryls to disulfides. DMPS is excreted at least partially intact, but its quantitative metabolic fate remains to be determined.

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ROLES OF DOPAMINE AND GABA IN NEUROTOXICITY OF ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS

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The toxicity of organophosphorus cholinesterase inhibitors is generally thought to be due to their irreversible inhibition of cholinesterase (1). It has been demonstrated that different cholinergic hyperactivity induced symptoms would be disrupted only if the cholinesterase activity in brain was below a certain critical level, e.g., 40% of normal (2,3). However, some of the behavioral changes induced by organophosphorus cholinesterase inhibitors do not completely correlate with the degree of cholinesterase inhibition (4-8). Therefore, whether all of the toxic symptoms are due to the derangement of cholinergic function or if other neurochemical changes might be, in part, responsible is unclear. The development of tolerance to organophosphorus cholinesterase inhibitors has also been well established as being a result of repeated administration of these inhibitors. During the past few years, numerous studies on these agents have resulted in understanding of how alterations of cholinergic hyperactivity might be involved in the tolerance phenomenon induced by these agents. The details have been summarized by Costa et al. (9) and Ho & Hoskins (10). Therefore, the present communication summarizes our recent studies of the effects of acute and subacute administrations of organophosphorus inhibitors on the acetylcholine, dopamine and GABA systems.

ACUTE TOXICITY AND THE DEVELOPMENT OF TOLERANCE TO ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS IN THE RAT: Organophosphorus cholinesterase inhibitor toxicity and tolerance in rats were studied (11-13). A single injection of (acute exposure to) diisopropylfluorophosphate (DFP) showed dose-dependent depressions in body weights and in food and water consumption. The animals recovered within 72 h. Daily injections of DFP (subacute exposure) caused significant depressions in all three parameters. However, tolerance to DFP in terms of growth rates, food and water consumption occurred. DFP induced behaviors (e.g. tremors, chewing-movements, hind-limb abduction and hypothermia) increased in a steeply dose dependent manner; all, except chewing-movements, subsided after 7 h. Subacute treatment with DFP for up to 1 month produced biphasic patterns of change for all the behavioral parameters. Tremor appeared in a complex spectrum of slow to intense fast types. Except for chewing-movements, tolerance developed for all these behaviors, but at different rates. The assessment of comparative toxicity of DFP, tabun, sarin and soman was also studied. The LD_{50} in rats for DFP, tabun, sarin and soman were 14.5, 1.9, 1.4 and 0.88 μmole/kg s.c., respectively. Our recent study further demonstrated that tolerance does develop to soman, sarin and tabun after daily administration of low doses. Therefore, the data show dose dependency of general toxicity during acute and subacute exposure to organophosphorus cholinesterase inhibitors and of tolerance during subacute exposure.

EFFECTS OF ACUTE AND SUBACUTE ADMINISTRATION OF DFP ON THE CHOLINERGIC SYSTEM: The effects of acute and subacute administration of DFP to rats on AChE activity (in striatum, medulla, diencephalon, cortex and cerebellum) and muscarinic receptor characteristics (in striatum) were investigated (14). After a single injection of DFP the striatal region was found to have the highest degree of AChE inhibition. After daily DFP injections, all brain regions had the same degree of AChE inhibition, which remained at a steady level despite the regression of the DFP induced cholinergic overactivity. Acute administration of DFP did not affect the muscarinic receptor characteristics; subacute administration of DFP for either 4 or 14 days reduced the number of muscarinic sites without affecting their affinity. The in vitro addition of DFP to striatal membranes did not affect muscarinic receptors. Our results also indicate that none of the lethal or sublethal doses of DFP, soman, sarin and tabun had any apparent effect on choline acetyltransferase (12).

EFFECTS OF ACUTE AND SUBACUTE ADMINISTRATION OF ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS ON THE DA SYSTEM: The effects of acute and subacute DFP treatment on striatal DA and its metabolite, dihydroxyphenylacetic acid (DOPAC), levels and DA receptor binding characteristics were studied (13,14). After acute treatment, striatal DA and DOPAC levels were altered and the DOPAC/DA ratios were consistently increased within the first 2 h. After subacute treatment for 4 and 14 but not for 28 days, both DA and DOPAC levels were decreased without a change in their ratios. Our studies further indicated that acute administration of DFP increased the number of DA receptors. Subacute treatment of DFP also caused an increase in the number of DA receptors after 14 days of treatment. However, this increase was considerably lower than that observed after the acute treatment. These data suggest that the changes in DA metabolism arose secondarily to an elevation of brain ACh following AChE inhibition. A prolonged change in the levels or turnover of DA could be responsible for increase of postsynaptic DA receptor density.

Recently, we also studied behavioral supersensitivity to atropine following treatment with organophosphorus cholinesterase inhibitors. Injection of DFP to rats produced characteristic behavioral effects such as tremors lasting for about 6 h. These effects were dose dependently blocked by atropine sulfate. Atropine treatment alone elicited, intermittently and in a dose dependent manner, rhythmic limb-shakes (myoclonus), bodyshakes and stereotyped sniffing, head movements and turning, but these behavioral changes were less prominent after DFP pretreatment within 6 h. However, in contrast, 24 h to 3 days after a single injection of DFP, these atropine induced responses were enhanced. Furthermore, the same effect occurred even when the animals had become behaviorally tolerant to DFP. Methylatropine blocked the peripheral symptoms of DFP, without any other effects. Similarly, the related agents, soman and sarin, led to behavioral super-sensitivity to atropine. The results suggest that muscarinic antagonists can precipitate exaggerated dopaminergic (stereotypies) and perhaps also serotonergic (myoclonus) types of responses following exposure to irreversible cholinesterase inhibitors.

EFFECTS OF ACUTE AND SUBACUTE ADMINISTRATION OF ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS ON THE GABA SYSTEM: The effects of acute and subacute injections of DFP to rats on GABA synaptic function were investigated in the striatal region of the brain (12,14,15). Acute as well as subacute treaments increased levels of GABA and its precursor (glutamate) and decreased GABA uptake and release. None of the treatments affected activity of GABA-transaminase. Glutamic acid dicarboxylase activity was increased by soman, sarin and tabun at certain lethal doses but was not affected by DFP even at the lethal dose.

Our results also showed that acute administration of DFP increased the number of GABA receptors without affecting their affinity. In subacutely-treated animals, DFP caused an increase in the number of GABA receptors after 14 days of treatment. This increase, however, was considerably lower than that observed after the acute treatment.

CONCLUSION: The results indicate an involvement of the GABAergic and dopaminergic systems in the action of organophosphorus cholinesterase inhibitors. It is suggested that the GABAergic and dopaminergic involvement may be a part of a compensatory inhibitory process to counteract the excessive cholinergic activity produced by these agents. Based upon the evidence presented we propose that GABAergic and dopaminergic neurons interact with cholinergic neurons after acute and subacute exposure to these agents.

Under normal circumstances (i.e., no exposure to organophosphorus cholinesterase inhibitors) a balance between excitatory cholinergic and the inhibitory GABAergic and dopaminergic activities is maintained in the striatum (basal ganglia).

After acute exposure to one of the anticholinesterase inhibitors, inhibition of AChE results in increased cholinergic activity which is balanced by increases in dopaminergic and GABAergic activities due to increases in DA and GABA receptor densities.

After subacute exposure to one of these inhibitors of AChE, in which synaptic concentrations of ACh are still high, the muscarinic receptor characteristics are changed such that there is a decrease in number of receptors as well as in receptor sensitivity to ACh. Thus, the effect of increased concentrations of ACh in this situation results in a "normal" cholinergic (excitatory) response that, in turn, is balanced by "normal" inhibitory responses of GABA and DA due to a return to normal of the inhibitory receptor populations.

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SEARCH FOR AN ENDOGENOUS DFPase IN MOUSE BRAIN

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Organophosphorus compounds are toxic substances that bind covalently to the active site of acetylcholinesterase (AChE) irreversibly inhibiting that enzyme, they include di-isopropylfluorophosphate (DFP), as well as the very potent nerve gasses soman, sarin and tabun. The recent discovery of an enzyme hydrolyzing these compounds in squid axons (1) led us to search for a similar enzyme in mouse brain. Besides being useful in inactivating unwanted stocks of these compounds, such an enzyme might prove ultimately to be of therapeutic value.

METHODS: Our basic approach was to incubate mouse brain homogenates, or subcellular fractions derived from the latter, with DFP at 37°C for various lengths of time. Following incubation, samples were centrifuged, and the supernatants analyzed for DFP activity remaining. DFP activity was assayed by incubating it with a microsomal (P_3) fraction from mouse brain, and determining its ability to inhibit AChE, as measured by the procedure of Ellman et al. (2). For every assay a standard curve was constructed using DFP of several known concentrations and this curve used to determine DFP concentrations in the supernatant samples.

In some experiments the binding of DFP to mouse brain fractions was also determined. ^{3}H-DFP was incubated with the fraction for various lengths of time, then the sample centrifuged and radioactivity associated with the pellet determined. In the case of non-particulate fractions, binding was determined by passage down a Sephadex G-50 column, which separates free ^{3}H DFP from that bound to high molecular weight material.

RESULTS: Incubation of DFP with mouse brain homogenates at pH 7.4 resulted in a time dependent decrease in DFP activity present in the supernatant, although a plateau was generally reached before all DFP was inactivated (Table 1). There was essentially no inactivation at 0°C. Inactivation also occurred in mitochondrial and microsomal fractions, with the plateau appearing much sooner.

The observed inactivation could result from two factors other than enzymatic degradation; spontaneous hydrolysis and binding of DFP to the fractions. Table 2 shows the spontaneous hydrolysis of DFP at 37°C and at 4°C. At 37°C, about 10% of the DFP is inactivated per hour with a higher degradative rate observed at pH 9 and a lower at pH 5. At 0°C, hydrolysis is negligible in 2 h, though quite significant over longer period.

Binding of ^{3}H DFP to brain fractions is shown in Table 3. When 10 mg of homogenate was inducated at pH 7.4 with DFP (1 mg), a maximum of about 5% of the radioactivity was associated with the pellet after 30 min; this declined slightly over longer incubation periods. Somewhat more binding was observed to P_2 and P_3 fractions which also showed a decline after 30 min. This decline is presumably due

TABLE 1: Inactivation of DFP by mouse brain fractions

Tissue	Time of incubation 15 min	30 min	60 min	2 h	2 h, 0°C
Homogenate (17 mg/ml)		0.18	0.26	0.59	
Homogenate (17 mg/ml)	0.057	0.060	0.078	0.080	0.009
Mitochondrial (4 mg/ml)	0.042	0.040	0.042	0.041	
Microsomal (2.5 mg/ml)	0.033	0.031	0.027	0.030	

The general procedure is described in Methods. (a) DFP (1.0 mg) incubated per ml; (b)-(d) DFP (0.1 mg) incubated per ml. Valves are mg DFP inactivated at 37°C, pH 7.4.

TABLE 2: Spontaneous inactivation of DFP

	30 min	1 h	2 h	1 day	2 days	3 days
pH 7.4	91	82	75			
pH 5.0	-	-	79			
pH 9.0	-	-	47			
pH 7.4, 4°C	-	-	99	58	49	44

DFP (0.1 mg) incubated in 1 ml buffer for various lengths of time, then its concentration assayed as described in Methods.

TABLE 3: Binding of ^{3}H-DFP to brain fractions

Sample	Incubation time 15 min	30 min	1 h	2 h
Homogenate	4.9	5.3	5.0	4.2
Mitochondrial	11.7	13.4	13.1	9.1
Microsomal	5.8	6.3	5.6	3.7

DFP (0.1 mg/ml) incubated with sample at 37°C and pH 7.4 for times indicated, then bound radioactivity determined as described in Methods. All values are % of total radioactivity recovered.

to aging which, as noted above, would result in the loss of bound radioactivity.

By adding together the amounts of DFP inactivated spontaneously, and that bound to brain fractions, one can calculate a theoretical value for DFP inactivated non-enzymatically. This has been done in Table 4, where all DFP bound values

TABLE 4: Theoretical rates of DFP inactivation due to spontaneous hydrolysis and binding and comparison with observed rates

Fraction	(A) DFP bound	DFP (B) Spontaneously hydrolyzed	(C) = (A) + (B) Theoretical inactivation	(D) Actual inactivation
0.90 ml Homog.	0.028±0.006	0.020±0.002	0.048±0.008	0.078±0.010
0.10 ml Homog.	0.007±0.002	0.020±0.002	0.027±0.004	0.035±0.004
0.50 ml P_2	0.026±0.005	0.020±0.002	0.046±0.007	0.042±0.005
0.50 ml P_3	0.012±0.004	0.020±0.002	0.032±0.006	0.026±0.004

All values are for 1 h incubation at 37°C, pH 7.4 and are expressed as the mean ± S.D. of two determinations (mg protein). Bound DFP is calculated as twice the % of radioactivity in pellets.

have been calculated by doubling the amount of radioactivity bound, to correct for possible aging. When these theoretical values are compared with the actual values, it is seen that in the case of the homogenates, a significant difference remains, whereas in the P_2 and P_3 fractions it does not.

DISCUSSION: Our results suggest that mouse brain homogenates contain some inactivating ability that cannot be completely ascribed to binding or spontaneous hydrolysis. Since this activity is not found in P_2 and P_3 fractions, it may be in the supernatant (S_3). We have since tested S_3 and indeed found it can inactivate DFP; there is negligible binding of ^{3}H-DFP to S_3, suggesting the inactivation is enzymatic. In preliminary experiments we have found that the activity is heat stable, partially but not completely lost by dialysis, and activated by Mn^{++}. Further work is in progress to characterize it.

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